THE DESTRUCTION OF RED CELLS BY ANTIBODIES IN MAN. II. PYROGENIC, LEUKOCYTIC AND DERMAL RESPONSES TO IMMUNE HEMOLYSIS[1]

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The immune destruction of red cells is usually mediated by the filtration of agglutinated, or agglutinable, red cells (1, 2). Initially red cell agglutination is enhanced in the circulation by the action of certain hydrophilic macromolecules, most notably fibrinogen, which induce rouleaux formation and red cell sedimentation (2, 3). Strongly, or coarsely, agglutinated red cells are largely trapped in the liver and lungs, whereas weakly, or finely, agglutinated cells are trapped mainly by the spleen (2). The greater proficiency of the spleen in filtering agglutinable, antibody-coated, red cells, as well as red cells which have been altered slightly in size or shape, may be based entirely upon the physical relation between the size of the cell particle and the pore size and perfusion pressure of essentially mechanical filters (4). The living filter beds of the reticuloendothelial system are presumably scoured by the phagocytic activity of macrophages, and indeed the lysis of red cells coated with nonhemolyzing (anti-D) antibodies appears to occur within a few minutes of their sequestration in the spleen (2).

In the course of making certain of the observations summarized above, which involved the injection of small volumes of incompatible red cells into normal subjects, an interesting pattern of host response to the injected cells emerged. It is well known that acute blood destruction may provoke an assortment of clinical reactions, including pain (particularly in the precordium and low back), dyspnea, coughing, flushing, chills, fever, nausea and vomiting, as well as certain allergic manifestations such as urticaria and even serum sickness (5). The pathogenesis of these phenomena, including the chills and fever of acute hemolytic episodes and the milder protracted fever which may attend chronic hemolytic states, is obscure. Marked alterations in blood leukocyte levels are also frequent in hemolytic disorders. Relatively little exact information exists concerning the acute phenomena partly because most observations have been made during transfusion reactions in which initial changes were not observed and in which the effects of contaminating bacterial pyrogens, shock, sensitivity to the buffy coat (6), and so forth could not be excluded. Nevertheless, an abrupt leukopenia was early found to occur during paroxysmal cold hemoglobinuria (7, 8), and leukopenia was observed following the injection of isoantibodies into dogs (9) and of incompatible blood into human subjects (10). More recently Swisher (11) described leukopenia and thrombocytopenia in dogs given incompatible blood in those instances where complement was fixed; and mixed agglutinates of red cells, leukocytes and platelets were noted, both *in vivo* (11) and *in vitro* (12). In the present studies, preliminarily reported elsewhere (13), fever and leukopenia were found to be characteristic of the normal host response to immune hemolysis and analogous in their pathogenesis to the host responses to other antigen-antibody reactions and to bacterial endotoxins.

METHODS

Techniques of preparing red cells and antibodies for injection. In most experiments whole blood was drawn into sterile syringes and transferred to screw-capped glass tubes containing one-fourth volume of acid-citrate-dextrose (ACD) solution. Following experimental manipulations *in vitro* the cells were washed twice in 10 to 20 volumes of physiologic saline before resuspension in saline and injection. The saline and anticoagulant solutions employed were sterile and demonstrated (in rabbits and/or in human subjects) to be pyrogen-free. Plasma contain-

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ing red cell antibodies was obtained by drawing whole blood into sterile plastic equipment containing either ACD or ethylenediaminetetracetic acid (EDTA) as anticoagulant; the blood was centrifuged and the plasma transferred to a second plastic bag via a closed system for storage at − 20° C. until used.[2] Again, the sterility and nonpyrogenicity of equipment and anticoagulant solutions were verified before use. Red cell sensitization with anti-D *in vitro* was achieved by incubating each volume of red cells in two volumes of physiologic saline with two volumes of anti-D plasma for one hour at 37° C., after which the cells were washed as described above.

Techniques involving Cr^{51}. In most experiments red cells were labeled with $Na_2Cr^{51}O_4$,[3] as described previously (2), before being washed as described above. The suspensions were injected intravenously into human subjects within a period of one minute. Thereafter specimens of blood were drawn periodically from each subject into saline-wet syringes and then were divided into two parts. One part, which was mixed with dry "balanced" oxalate, was later lysed by freezing and thawing to provide samples for measuring whole blood radioactivity. The other part, mixed with one-tenth of its volume of 3 per cent sodium citrate solution, was centrifuged, and the supernatant plasma recentrifuged, to provide plasma for determination of its radioactivity. The radioactivity of 3 ml. samples of whole blood, plasma and the Cr^{51} standards was determined in a well-type scintillation counter. The distribution of radioactivity at body surface over the subjects' hearts, livers and spleens was determined with a directional scintillation counter as described elsewhere (14).

Leukocyte measurements. Leukocytes were counted by standard techniques (15) in samples pipetted from oxalated blood specimens drawn within a few seconds of venesection. The pipetting was performed promptly for theoretical reasons, although enumeration of leukocytes pipetted from the same oxalate specimens two to three hours later in several experiments revealed that their leukocyte counts remained constant within that period of time *ex vivo*. Differential leukocyte counts were made on cover slip smears of blood obtained from the needle tip and stained with Wright's stain. Unless a scarcity of cells prevented it, 2,000 leukocytes were examined during each differential count.[4] Direct eosinophil counts were made, using Randolph's solution (15).

Miscellaneous techniques. Body temperatures were determined rectally at frequent intervals during base line (control) measurements, with the subject resting in bed for 30 minutes or more, and during the experimental period. Plasma hemoglobin levels were determined by a photo-electric adaptation of the benzidine technique (15), using the same specimens employed for measuring plasma Cr^{51}. The Coombs (antiglobulin) test (16) was performed by the tube technique and the polyvinylpyrrolidone ("P. V. P.") test was carried out as described elsewhere (3). Peripheral blood was searched for the presence of "autoagglutination" by suspending 0.1 ml. of fresh or oxalated blood in 10 ml. of warm saline, and microscopically examining a large drop of this freely-moving cell suspension on a glass slide. The osmotic fragility of red cells (17), the serum bilirubin levels (18), and plasma fibrinogen levels (19) were determined in certain observations.

Human subjects. The normal human subjects who cooperated in this study were either elderly men with minor illnesses or laboratory personnel, in whom no diseases of the hematopoietic or reticuloendothelial systems were believed to be present. Several other subjects were studied who had previously undergone accidental immunization against the D antigen of the Rh system as a result of transfusion or pregnancy.

RESULTS

Effects of ABO-incompatible red cells

The injection of 10 ml. of washed type A or type B red cells into an ABO-incompatible recipient (whether or not he possessed demonstrable isohemolysins) led to the sequestration, largely in the liver, of most of the injected red cells within a few minutes, as determined by measurement of blood and body surface Cr^{51} activity. Simultaneously, as revealed in Figure 1, blood leukocyte levels abruptly fell to about 30 per cent of control levels, remained low for approximately 30 minutes, and then rose to levels somewhat above control levels in two to four hours. Differential leukocyte counts, as in a characteristic study depicted in Figure 2, revealed that leukopenia represented a virtual disappearance of granulocytes and of monocytes from the circulation. In general the levels of mature granulocytes fell more sharply than did those of band forms. The influence of granulocyte maturity upon the granulocytopenic effect of isohemolysis was most clearly evident in a study of a patient with chronic myelogenous leukemia. The injection of ABO-incompatible red cells into this patient caused a pronounced reduction in the level of mature granulocytes, a moderate fall in the level of band forms, but had no effect on the levels of metamyelocytes and myelocytes. In most subjects, levels of mature eosinophils (not shown), measured both directly and indirectly, fell as dramatically as did those of mature neutrophilic

[2] We are grateful to Dr. A. R. Jones and the Blood Grouping Laboratory of Boston, Mass., for their cooperation throughout this study in providing the antisera.

[3] "Rachromate" of high specific activity, Abbott Laboratories, North Chicago, Ill.

[4] We are indebted to Miss Geneva A. Daland, who performed most of these differential counts.

granulocytes. In several experiments basophil levels (not shown) also fell, although the number of cells involved was often too small for certainty. Within 20 minutes of the disappearance of granulocytes and monocytes, neutrophilic band forms (and small numbers of metamyelocytes) began to increase in the circulation, reaching peak levels within two hours of 5 to 20 times preinjection levels, and falling slowly thereafter as an adult neutrophilia predominated. Meanwhile, there was a relatively small rise in eosinophils, and monocyte levels increased and rose very slowly to preinjection levels without an overshoot. Beginning about two hours after the injection of red cells, a moderate decline in lymphocytes was often observed, as in Figure 2, reaching a low point in four hours.

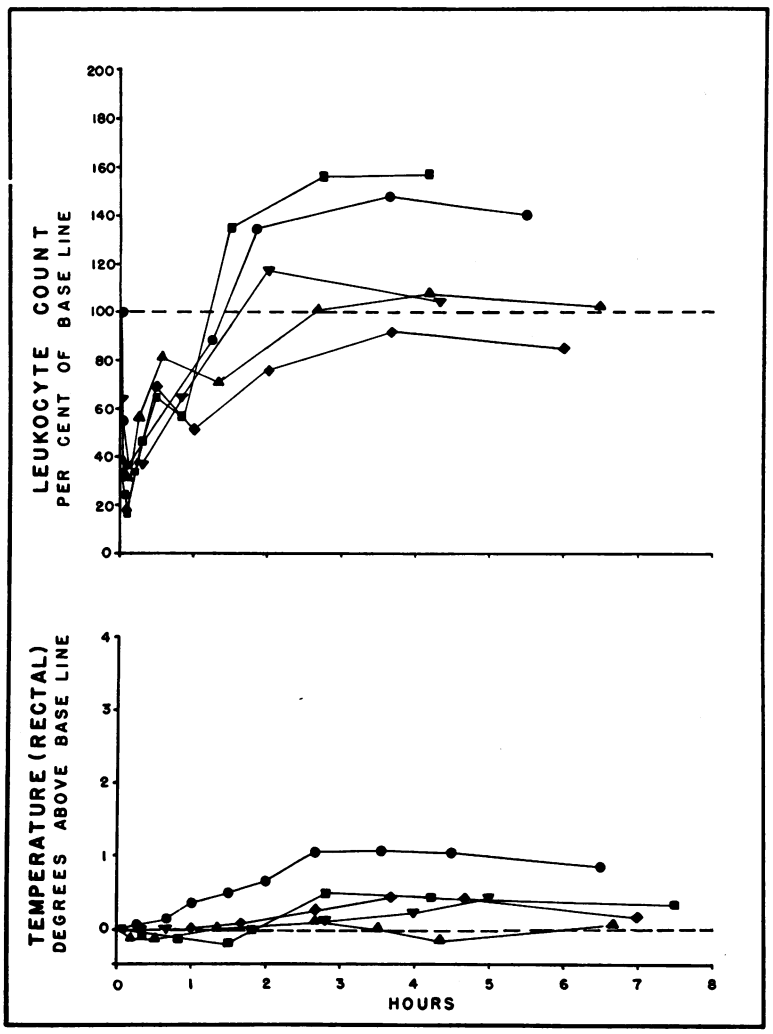

FIG. 1. LEUKOCYTE AND BODY TEMPERATURE CHANGES FOLLOWING INTRAVENOUS INJECTION OF ABO-INCOMPATIBLE RED CELLS

As shown in the upper portion of the figure, blood leukocyte levels of the five normal subjects fell precipitously to about 30 per cent of base line values within two to three minutes. Leukopenia was rather transient and normal, or above normal; leukocyte levels were regained within two or three hours. No chills were observed, and, as shown in the lower portion of the figure, the subjects' rectal temperatures tended to increase only slightly to maximum levels in three or four hours.

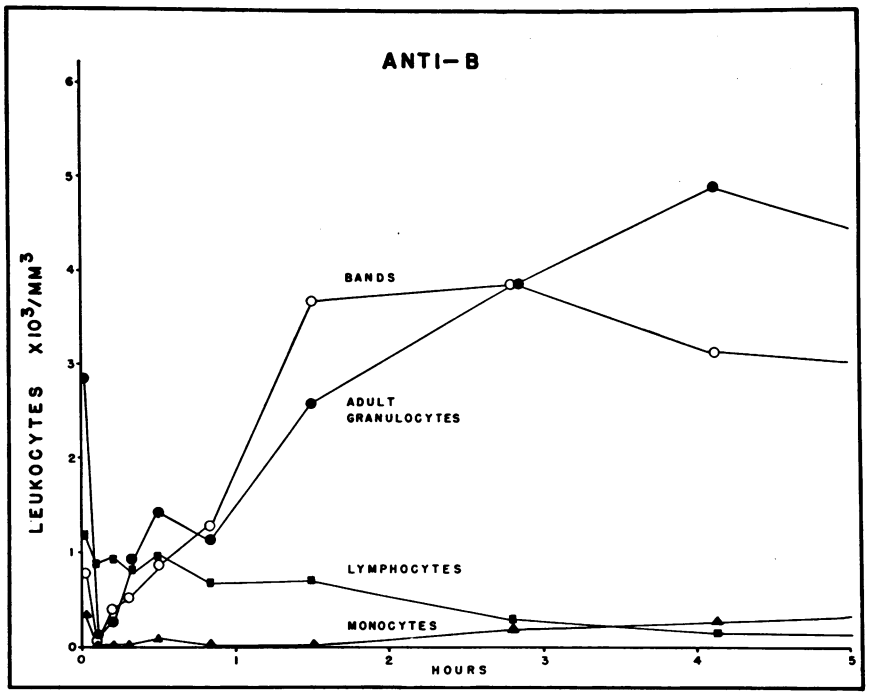

FIG. 2. CHANGES IN LEUKOCYTE DIFFERENTIAL COUNTS FOLLOWING INTRAVENOUS INJECTION OF TYPE B RED CELLS INTO A TYPE O SUBJECT

In this representative study, the abrupt leukopenia portrayed in Figure 1 is seen to result from a virtually complete disappearance of granulocytes and monocytes. Within 30 minutes of the red cell injection, the numbers of band forms began to increase and within two hours reached levels fivefold higher than base line. At the same time adult granulocytes increased to reach mildly elevated levels in about four hours. Note that a monocytosis failed to appear, and that the lymphocyte count was unaffected during the acute hemolysis, but began to decline gradually two hours after the injection.

Usually the leukopenia produced by ABO-incompatible red cells was maximal between three and five minutes after injection. Speed of onset was observed more critically in subjects having an inlying cardiac catheter and a Cournand needle lying in the brachial artery, which thus permitted samplings at approximately 10 second intervals of pulmonary artery blood and brachial artery blood, respectively.[5] One of three such studies is presented in Figure 3. After the catheters had been in place for 30 minutes or more, 10 ml. of Cr^{51}-labeled ABO-incompatible red cells was injected into a leg vein. Leukocyte levels began to fall within a few seconds of the appearance of Cr^{51}-labeled red cells at the sampling sites. There was no definite difference at a given instant between leukocyte levels in the pulmonary artery and in the brachial artery.

The number of ABO-incompatible red cells necessary to produce a definite leukopenia on intravenous injection was determined by injecting several normal subjects with various amounts of red cells which had been freshly drawn in heparinized syringes from incompatible donors and washed twice with saline. As seen in Table I, relatively small numbers of red cells (2.2×10^{10} red cells or less than 2.0 ml. of packed cells) caused a 65 per cent reduction in leukocyte levels and an almost total disappearance of granulocytes. Smaller injections caused lesser degrees of leukopenia, but as few as 5×10^{8} red cells (0.05 ml. of packed cells) caused a significant fall in leukocyte levels. By estimating the number of circulating leuko-

[5] Studies on patients undergoing catheterization of the right side of the heart were possible through the generous cooperation of Dr. Walter Abelmann and his associates.

cytes in these subjects, it was calculated that as many as 15 leukocytes, chiefly granulocytes, may be removed from the recipient's circulation for each incompatible red cell injected. A 10 ml. volume of type O serum, having an agglutinin titer of $\frac{1}{128}$ against a 5 per cent suspension of type A cells and a hemolysin titer of $\frac{1}{4}$, was injected intravenously into a type A normal subject. There

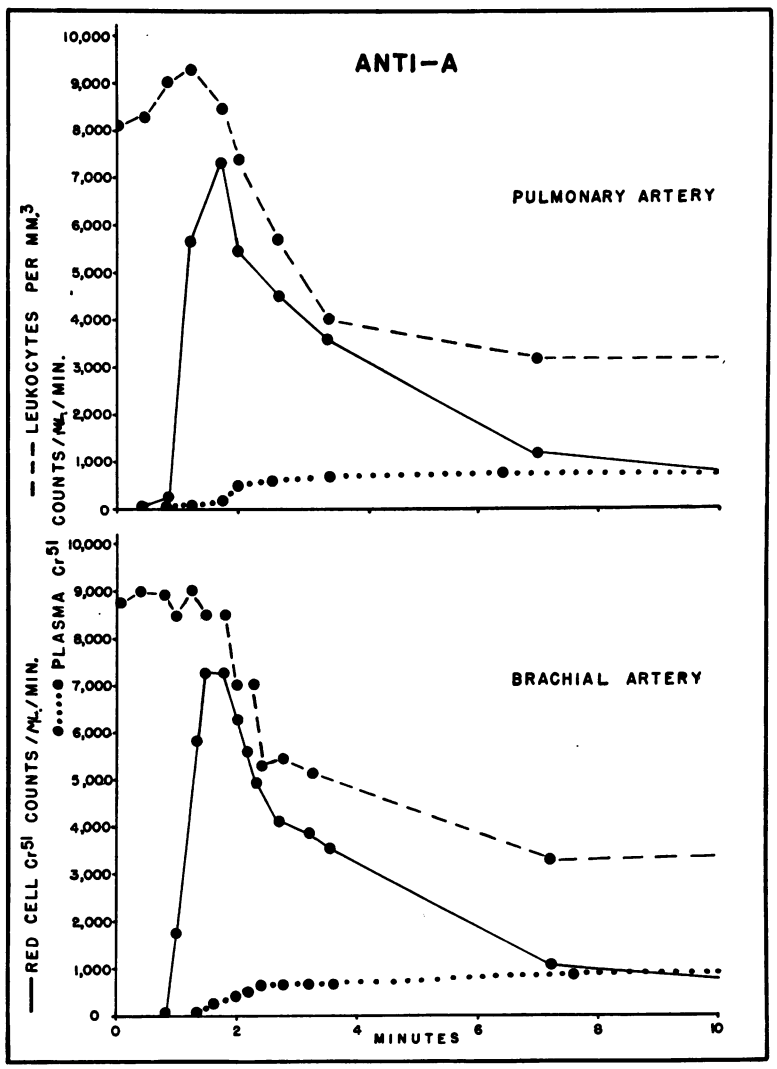

FIG. 3. A COMPARISON OF LEUKOCYTE LEVELS AND RED CELL AND PLASMA RADIOACTIVITY IN PULMONARY ARTERY AND BRACHIAL ARTERY FOLLOWING THE INJECTION OF Cr^{51}-LABELED TYPE A RED CELLS INTO A TYPE O SUBJECT

Within less than a minute of the first appearance of Cr^{51}-labeled red cells at the sampling sites, and within a few seconds of the appearance of Cr^{51} (and of hemoglobin) in the plasma, leukocyte levels (interrupted line) commenced to fall. There was no certain difference in leukocyte levels of blood before and after passage through the lungs. At both sites, leukopenia (interrupted line), Cr^{51}-labeled red cell disappearance (uninterrupted line), and hemoglobinemia (dotted line) became evident almost simultaneously. Note that in this subject, whose serum revealed no isohemolysins on conventional testing *in vitro*, most of the Cr^{51} derived from the incompatible red cells failed to appear in the plasma, indicating that red cell sequestration was the chief mechanism of red cell destruction (2).

TABLE I

The comparative effects on leukocyte levels of intravenous injections of various quantities of ABO-incompatible red cells

Red cell type		Number of red cells injected	Reduction in leukocyte level (*% of base line*)	Time of maximum leukopenia (*min.*)	Estimated ratio white cells removed/red cells injected
Donor	Recipient				
B	A	5.0×10^8	−22	3	14.6
A	O	2.5×10^9	−37	3	6.0
B	A	8.8×10^9	−49	5	2.3
A	O	2.2×10^{10}	−65	5	1.3
B	O	1.2×10^{11}	−70	5	0.2

was no hemolysis or agglutination *in vivo,* presumably because of distribution of the antibodies among an excess of the antigenic red cells, and there was no leukopenia. However, when this same volume of serum was previously mixed *in vitro* with 1 ml. of the subject's type A red cells and the hemolyzed suspension was then injected, an immediate leukopenia followed.

The injection of 10 ml. volumes of Cr^{51}-labeled, washed ABO-compatible red cells into three normal subjects had no effect upon leukocyte levels, nor did leukopenia occur when similar volumes of compatible red cells were injected after they had been hemolyzed by repeated, rapid freezing and thawing. Solutions of hemoglobin derived from water-hemolyzed compatible red cells also had no effect upon leukocyte levels in two normal subjects. In a type O subject, however, the injection of the membranes of 10 ml. of type A red cells, after these cells had been hemolyzed by freezing and thawing and the membranes washed almost free of hemoglobin, caused a marked leukopenia. Several hematologically normal subjects were injected with a preparation of soluble A substance.[6] Six out of seven of the type O subjects developed a definite leukopenia, maximal in three minutes, whereas none of five type A subjects developed a leukopenic response. As with the injection of type A cells, the leukopenia caused by the injection of A substance resulted from a reduction in circulating granulocyte and monocyte levels.

As shown in the lower portion of Figure 1, the injection of ABO-incompatible red cells caused mild increases in the rectal temperatures of the recipients, with an average temperature maximum in five subjects of 0.5° F. above the pre-experimental level between three and four hours after injection. None of these subjects developed chills. In a few subjects transient symptoms appeared within two or three minutes of the injection of incompatible red cells. These symptoms included lumbar pain, dyspnea, hyperperistalsis, emotional instability and, in two instances, marked flushing of the face. In one type O subject transient dyspnea and flushing followed the injection of soluble A substance.

Effects of type D red cells in the presence of anti-D

After the injection of 10 ml. volumes of Cr^{51}-labeled, washed type D red cells into four otherwise normal subjects who possessed incomplete (nonagglutinating) antibodies against type D red cells, these cells were removed from the circulation, but more slowly than was the case with ABO-incompatible red cells. The labeled red cells were largely trapped in the spleen, following which plasma hemoglobin levels increased only slightly and gradually to a maximum about one hour after injection (2). Meanwhile, blood leukocyte levels declined slowly, but profoundly, to their lowest values at the end of about one hour (Figure 4). As with ABO-incompatibility, the leukopenia directly reflected a fall of granulocytes and monocytes, often to the point of their temporary disappearance (Figure 5). Thereafter, band neutrophils (along with younger forms) markedly increased, and later a moderate adult neutrophilia was seen, becoming maximal four to five hours after injection. A striking lymphopenia characteristically began about two hours after injection, reaching lowest values one or two hours thereafter.

A series of observations was then conducted in which normal Cr^{51}-labeled type D red cells were sensitized *in vitro* as was described above and re-

[6] "Blood Group Specific Substance A," derived from porcine gastric mucosa, Knickerbocker Blood Bank, New York, N. Y.

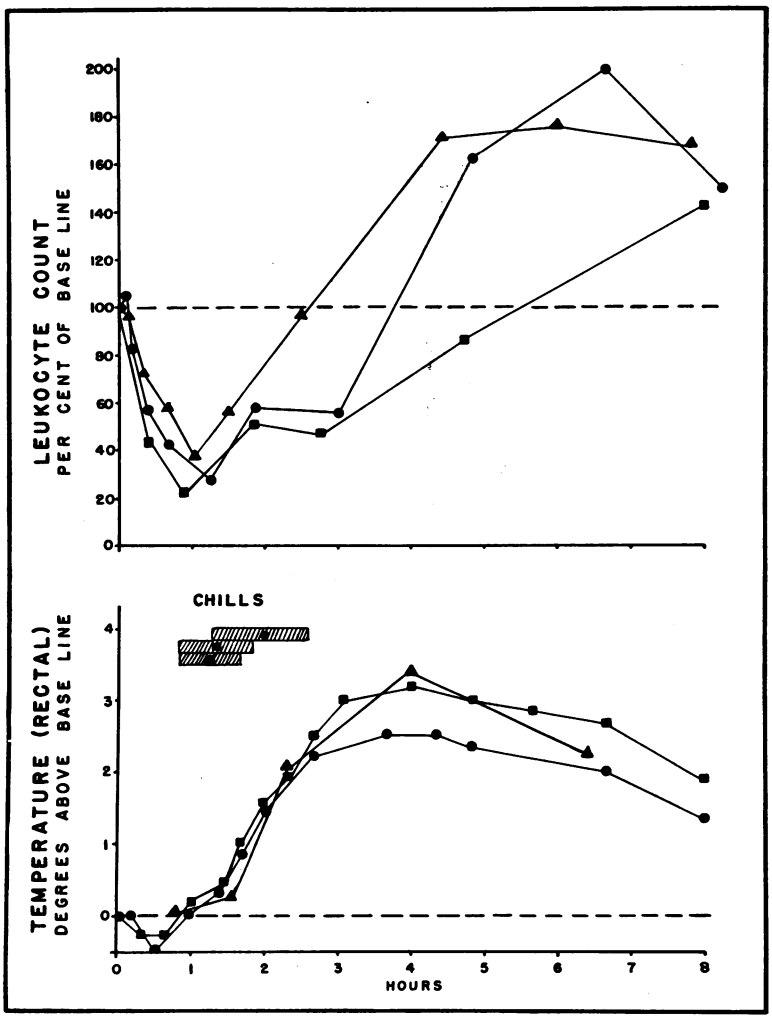

FIG. 4. LEUKOCYTE AND BODY TEMPERATURE CHANGES FOLLOWING INTRAVENOUS INJECTION OF TYPE D RED CELLS INTO D-SENSITIZED SUBJECTS

The upper portion of this figure depicts the resulting slow but marked reduction in the leukocyte levels of each of three subjects whose serum contained incomplete anti-D. Note the characteristic timing of the pyrogenic response. (Similar leukopenic and febrile responses occurred in a fourth subject; however, systematic observations of body temperature levels were not made.)

injected into the donor unless otherwise stated. These washed sensitized red cells were removed from the circulation of the normal D-positive subjects at a rate similar to that of type D red cells injected into sensitized subjects, and in this instance appeared to be sequestered exclusively in the spleen (2). Once again, as seen in Figure 6, a slow but marked depression of leukocyte levels occurred, maximal at one hour, following which a moderate neutrophilia developed and reached peak levels four to five hours after injection. As in the previous observations, the leukopenia resulted from a fall in granulocytes and monocytes, and was followed by a band neutrophilia, and two hours after injection by lymphopenia. A virtually identical sequence of events followed the injection of anti-D sensitized red cells into D-negative individuals, whereas the injection of D-negative red

cells labeled with Cr^{51} and incubated with anti-D plasma in the same fashion had no effect upon leukocyte levels.

The temperature responses of D-sensitized subjects receiving type D red cells, and of normal subjects receiving autogenous type D red cells previously sensitized with anti-D *in vitro,* are presented in the lower portion of Figures 4 and 6. No abrupt symptoms, such as occurred in some patients injected with ABO-incompatible red cells, were observed. However, in each situation, and without exception, chills developed between 50 and 75 minutes and lasted for 30 to 60 minutes. The chills were preceded by mild hypothermia, and were often accompanied by nausea, restlessness and occasionally emotional instability. This period was followed by a steady rise in rectal temperatures, to maximum values averaging 3° F. above initial levels between four and five hours after injection.

The intravenous injection of 20 ml. of anti-D plasma into three type D individuals led to smaller rises in plasma hemoglobin and to lesser reductions in leukocyte levels than were observed in the same individuals when 10 ml. volumes of their red cells were sensitized with 20 ml. of anti-D plasma *in vitro* and then reinjected. The intravenous injection of anti-D plasma into an unsensitized D-negative subject had no effect upon his leukocyte levels or temperature, as depicted in the left-hand portion of Figure 7. When, however, type D red cells were then injected about two hours later leukopenia promptly began and was followed by a pyrogenic response. Conversely, as shown in the right-hand part of Figure 7, type D red cells had no discernible effect when injected alone into another unsensitized D-negative subject. However, injection of anti-D plasma about two hours later promptly initiated leukopenia followed later by fever.

In a single experiment, 10 ml. of Cr^{51}-labeled type D red cells was added with sterile precau-

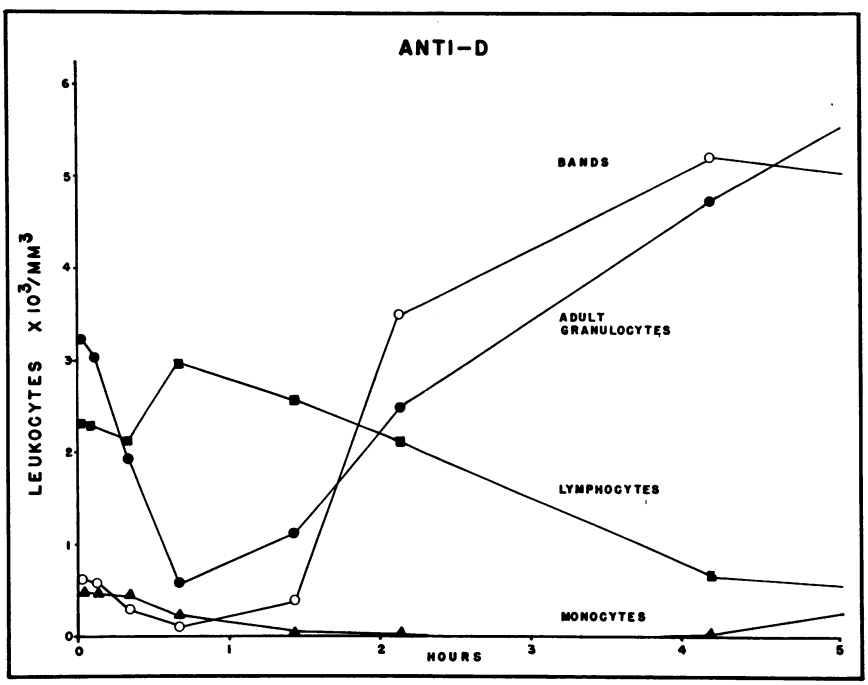

FIG. 5. CHANGES IN LEUKOCYTE DIFFERENTIAL COUNTS FOLLOWING INTRAVENOUS INJECTION OF TYPE D RED CELLS INTO A SENSITIZED D-NEGATIVE SUBJECT

As in ABO-incompatibility, striking reductions in granulocytes and monocytes accompanied red cell sequestration, whereas lymphopenia was delayed until two or more hours afterward. Band forms increased markedly in number, reaching eight times the initial value four hours after injection. As in Figure 2, the recovery of monocyte levels was slower than that of the granulocytes and there was no later monocytosis.

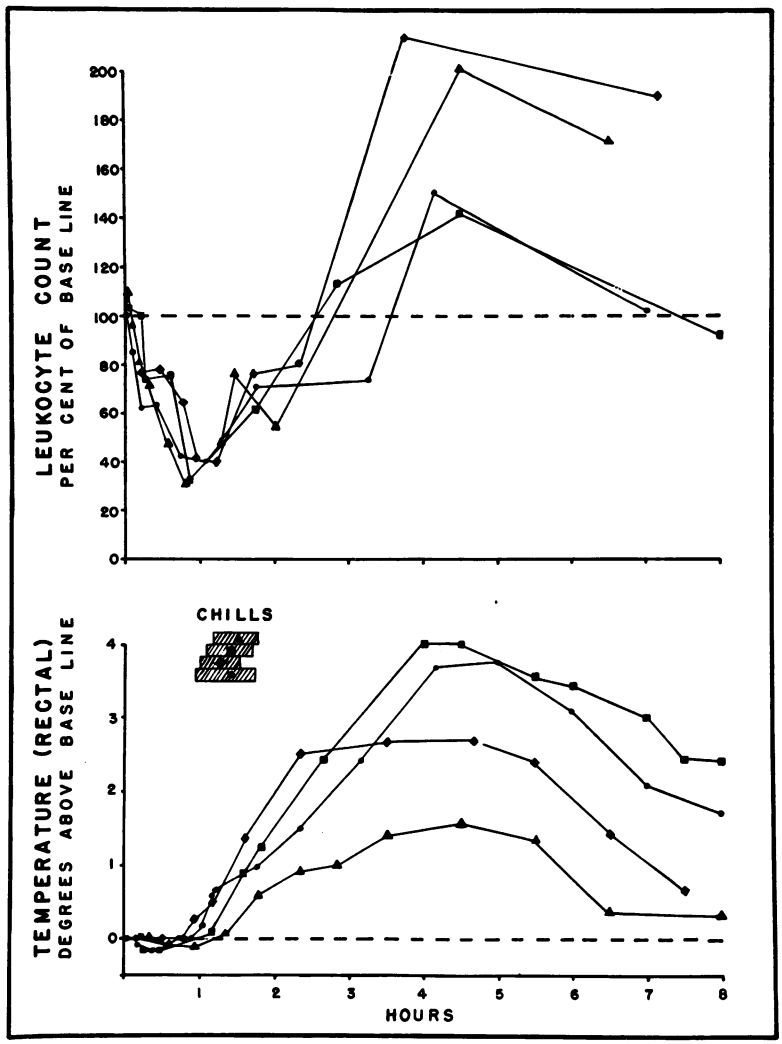

FIG. 6. LEUKOCYTE AND BODY TEMPERATURE CHANGES FOLLOWING INTRAVENOUS INJECTION INTO NORMAL SUBJECTS OF AUTOGENOUS TYPE D RED CELLS PREVIOUSLY SENSITIZED *IN VITRO* WITH ANTI-D

A slowly-evolving leukopenia followed by leukocytosis and a rather severe pyrogenic reaction is evident in each of four subjects. The average leukocyte and temperature responses following injection of sensitized autogenous red cells are very similar to those following injection of type D red cells into sensitized subjects (Figure 4).

tions to a plastic bag containing 500 ml. of freshly-drawn, whole blood from a D-negative subject. To this mixture was added 20 ml. of anti-D plasma and the suspension was incubated at 37° C. for two hours. The entire mixture was then injected into the D-negative subject in a period of six minutes. The subsequent rate of Cr^{51}-labeled red cell sequestration, the leukopenia, and the pyrogenic response were quite similar in extent and in timing to those experiments portrayed in Figure 6.

In both the experiments with ABO-incompatible red cells and the type D red cells exposed to anti-D, the rates at which red cell sequestration occurred were very similar to the rates at which leukopenia developed. In Figure 8, mean values of red cell survival, leukocyte levels, and plasma hemoglobin levels following injection of such

red cell suspensions, are portrayed for ready comparison.

The influence of several agents on the leukopenia and pyrogenic response to incompatible blood was examined. Two subjects were injected with sufficient heparin to prolong the clotting times of their blood in glass tubes to approximately one hour. Thereafter these subjects were injected with ABO-incompatible red cells and anti-D sensitized autogenous red cells, respectively. However, the heparin did not alter the leukopenic response to either injection, or the pyrogenic response to the sensitized cells. One subject was given 1 Gm. of aspirin one hour before the injection of 10 ml. of anti-D sensitized autogenous red cells and 1 Gm. of aspirin every two hours thereafter for six hours. The characteristic pattern of sequestration of the injected red cells, of the plasma hemoglobinemia, and of the leukopenia were unaffected; however, no chills were observed and only a slight rise in rectal temperature appeared during the six hour observation period. Two subjects were given 50 mg. of cortisone orally every six hours for 24 hours and 48 hours, respectively, before the injection of 10 ml. of anti-D sensitized autogenous red cells. In both instances there was an apparent suppression of the usual pyrogenic response, although the rate and site of sequestration of the sensitized red cells, and the subsequent leukopenia, occurred as it had in subjects untreated with cortisone.

Five subjects receiving 10 ml. of ABO-incompatible red cells, and three subjects receiving anti-D sensitized autogenous red cells were observed for alterations in blood platelet and plasma fibrinogen levels. In neither group did platelet or fibrinogen levels fluctuate significantly during the periods of observation.

Effects of red cells altered by nonimmunological mechanisms

Normal red cells were altered *in vitro* under sterile conditions by several kinds of nonimmunologic procedure. Unless otherwise stated below,

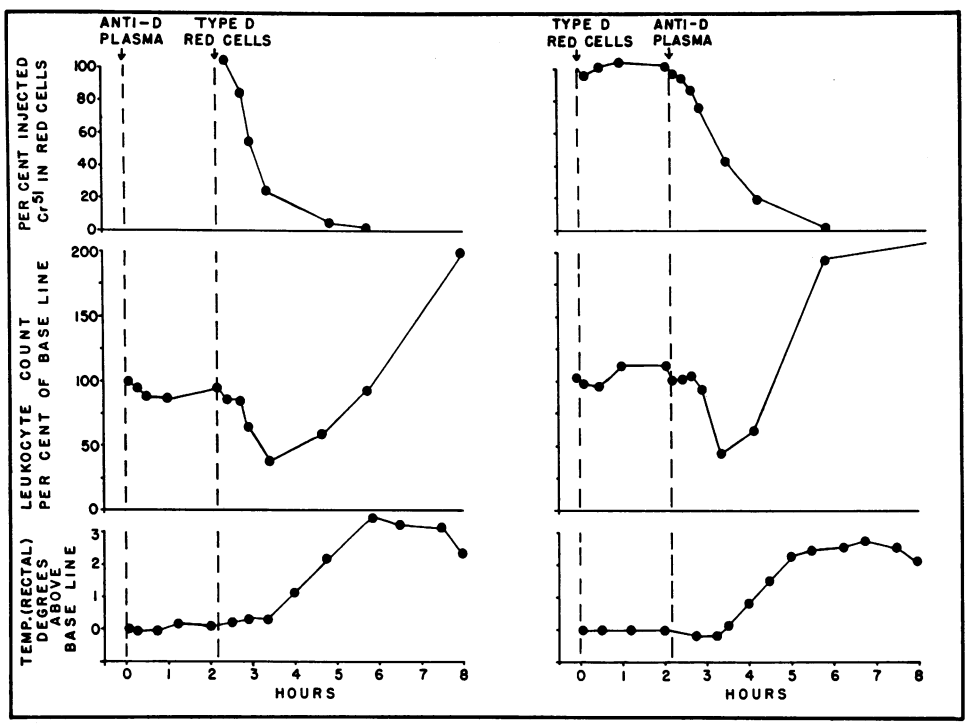

FIG. 7. RED CELL SEQUESTRATION, LEUKOPENIA, AND FEVER ON INTERACTION BETWEEN ANTI-D AND TYPE D RED CELLS IN THE CIRCULATION OF UNSENSITIZED D-NEGATIVE SUBJECTS

Note that leukopenia and fever failed to occur when either anti-D plasma or Cr^{51}-labeled type D red cells were given individually. When, however, both coexisted, the labeled red cells were rapidly removed from the circulation (upper portion), leukopenia developed (middle portion), and fever, initiated by chills, ensued (bottom portion).

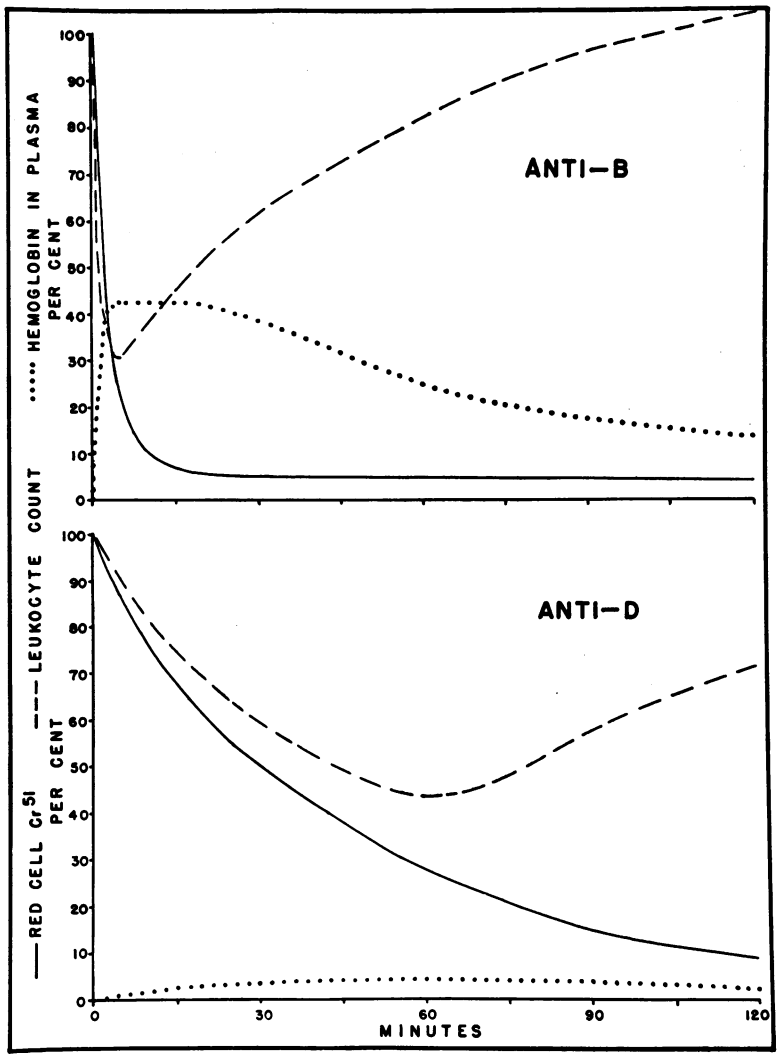

FIG. 8. COMPARISON BETWEEN THE RATES OF SEQUESTRATION OF INCOMPATIBLE TYPE B AND TYPE D RED CELLS AND THE ASSOCIATED APPEARANCE OF LEUKOPENIA AND HEMOGLOBINEMIA

In each portion of the figure the lines represent average values of the data obtained from several different subjects. The rapid sequestration of Cr^{51}-labeled type B red cells injected into five normal subjects whose serum contained anti-B (upper portion) was promptly followed by the development of leukopenia and hemoglobinemia. Similarly, the more slowly developing leukopenia and hemoglobinemia following the injection of Cr^{51}-labeled type D red cells into four sensitized, D-negative subjects (lower portion) occurred simultaneously with the sequestration of these cells. Leukocyte levels (interrupted lines) are expressed as per cent of initial levels, and red cell Cr^{51} (uninterrupted lines) and plasma hemoglobin levels (dotted lines) are expressed as the per cent of that injected.

the cells were labeled with Cr^{51} after the alteration was produced. After reinjection of the altered, Cr^{51}-labeled red cells into their normal donors, these subjects were studied as usual in terms of Cr^{51}-labeled red cell survival, plasma levels of hemoglobin and of Cr^{51}, body distribution of Cr^{51}, leukocyte levels, and body temperature. The results are summarized in Table II.

TABLE II

Observations on the destruction of red cells altered by nonimmune mechanisms

Method of producing red cell alteration	Survival *in vivo* of altered red cells		Hemoglobinemia (>4 mg. %)	Site of sequestration	Systemic effects	
	% sequestered	Half-survival			Fever	Leukopenia
Sterile incubation at 37° C.						
4 hrs. (ACD)*	0		0		0	0
24 hrs. (ACD)	30% (in 20 min.)		0	Liver	0	0
48 hrs. (ACD)	100% (in 40 min.)	4 min.	0	Liver	0	0
48 hrs. (defibrinated)	100% (in 60 min.)	6 min.	0	Liver	0	0
Acetylphenylhydrazine	100% (in 5 days)	8 hrs.	0	Spleen	0	0
Lecithin	68% (in 60 min.)	22 min.	0	Spleen	0	0

* Acid-citrate-dextrose.

A. Sterile incubation. Fifty ml. of normal whole blood mixed with ACD solution was stored at 37° C. for four hours without agitation. The Cr^{51} survival of the derived red cells on subsequent reinjection into the donor was unaffected, and there were no systemic reactions. Fifty ml. of normal whole blood incubated for 24 hours under these conditions showed no visual evidence of autohemolysis. There was a moderate symmetrical shift to the left in the osmotic fragility curve. The P. V. P. test was negative. On reinjection, 30 per cent of these red cells were rapidly sequestered, chiefly by the subject's liver. However, there was no rise in plasma hemoglobin or of plasma Cr^{51}, and no systemic reactions occurred. Normal whole blood (50 ml. amounts) which had been either mixed with ACD solution or defibrinated and then incubated for 48 hours under the conditions described above showed very little autohemolysis. There were moderate increases in methemoglobin concentration, in mechanical fragility, and the most osmotically fragile cells became more fragile, the least fragile cells became less fragile, while the mean osmotic fragility was relatively unchanged. The P. V. P. test was negative. In each instance these cells were abruptly removed from the circulation after their injection, and were sequestered largely in the liver; half-survival times were four minutes for red cells in ACD and six minutes for red cells from defibrinated blood. One of these two studies is presented in Figure 9. Despite the rapidity and completeness of this sequestration, there was no hemoglobinemia (all levels were below 3.5 mg. per cent), no Cr^{51} appeared in the plasma, and there were no febrile reactions or leukopenia. That the injected red cells were actually hemolyzed at the site of their hepatic sequestration, however, was suggested by a rise in indirect-reacting bilirubin, which began about one hour after injection.

B. Acetylphenylhydrazine. Normal red cells which had been labeled beforehand with Cr^{51} were exposed to a sterile solution of 1 per cent acetylphenylhydrazine for one hour at 37° C., and then washed once prior to reinjection into the donor. Although there was no supernatant hemoglobin in this preparation, much of the intracorpuscular hemoglobin had been precipitated as Heinz bodies (an average of four Heinz bodies per red cell) or had been converted to methemoglobin and sulfhemoglobin (54 per cent and 4 per cent of the unprecipitated hemoglobin, respectively). The P. V. P. test was negative. These red cells on reinjection were removed from the blood stream at a gradually diminishing rate, with a half-survival time of eight hours, and were sequestered almost entirely in the spleen. Again no hemoglobin or Cr^{51} was released into the plasma, and no febrile or leukopenic response occurred.

C. Lecithin. The red cells from 35 ml. of normal whole blood in 12.5 ml. of ACD solution were labeled with Cr^{51}, washed with physiologic saline, and then suspended in a 1 per cent sterile solution of animal lecithin at 25° C. for one hour. After rewashing, these lecithinized red cells were spherocytic in appearance under the microscope and their osmotic fragility was found to be symmetrically "shifted to the left," with the mean cellular fragility increased from 0.41 Gm. per cent sodium chloride before treatment with lecithin to

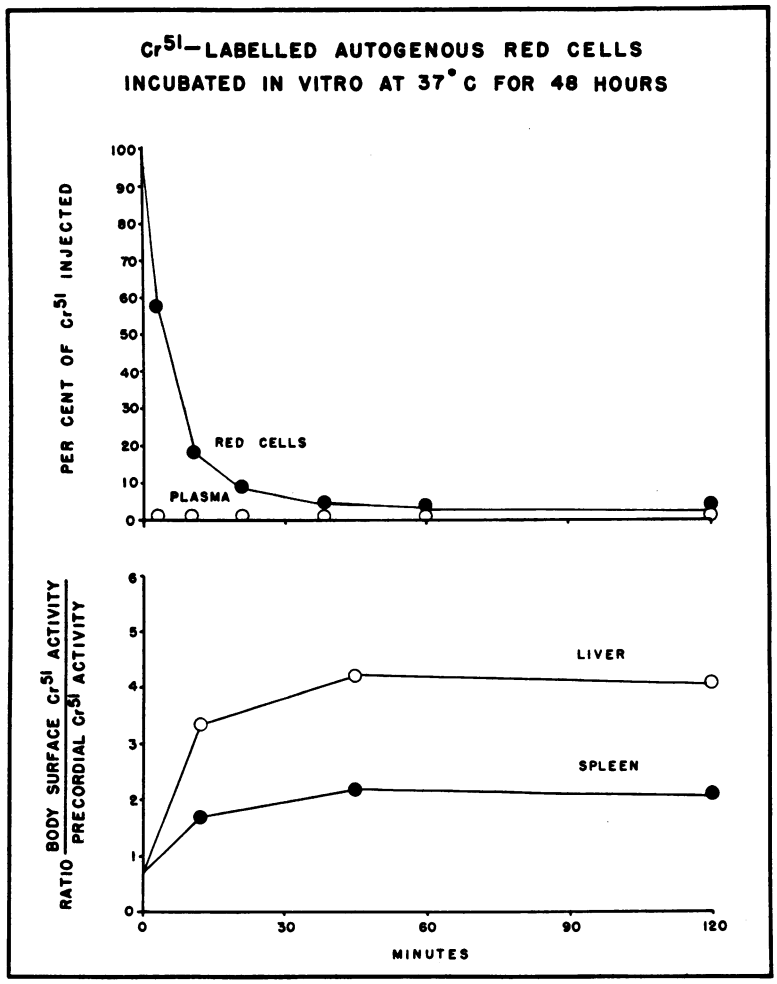

FIG. 9. THE RAPID HEPATIC SEQUESTRATION OF RED CELLS AFTER THEIR PROLONGED INCUBATION AS STERILE DEFIBRINATED BLOOD *IN VITRO*

Despite the extreme rapidity of red cell sequestration by the liver, comparable in rate and site to that seen with ABO-incompatibility, there was no hemoglobinemia. That an elution *in vivo* of the Cr^{51}-label did not ensue is indicated by the failure of Cr^{51} to appear in the subject's plasma. There was no change in blood leukocyte levels or in body temperature of either subject who received red cells altered by incubation *in vitro* for 48 hours.

0.62 Gm. per cent afterwards. Lecithinization increased the mean corpuscular volume from 86 to 103 μ^3 and the mean corpuscular hemoglobin concentration was decreased from 31 to 28 per cent. On reinjection into the donor, about two-thirds of the lecithinized spherocytes were sequestered in the spleen (half-survival, 22 minutes). Thereafter circulating red cell Cr^{51} slowly increased from 32 per cent of the injected dose in one hour to 57 per cent of the injected dose at the end of 24 hours (declining at a normal rate thereafter) while splenic radioactivity behaved in a reciprocal fashion (Figure 10). During the acute and, in part, transient [7] sequestration of lecithinized spherocytes by the patient's spleen, there was no rise in plasma

[7] Although one generally assumes that splenic sequestration of red cells causes their injury or destruction, it appears that these transiently trapped spherocytes were in some manner rejuvenated. Incubation for eight hours at 37° C. *in vitro* of lecithinized red cells in normal heparinized plasma failed to improve their osmotic fragility.

hemoglobin or Cr^{51}, and there were no changes in the patient's leukocyte levels or body temperature.

Observations in vitro and in the tourniquet-obstructed arm

The effects of red cell isoantibody interactions on leukocytes *in vitro* were studied by incubating various combinations of red cells, compatible and incompatible plasmas, and leukocytes. Leukocyte-rich suspensions were obtained by first removing red cells from whole blood by light centrifugation and then gently spinning down the leukocytes, as well as some of the platelets, from the supernatant plasma. Siliconized glassware was employed throughout and experiments were conducted in various systems involving plasma containing heparin or EDTA, plasma obtained by

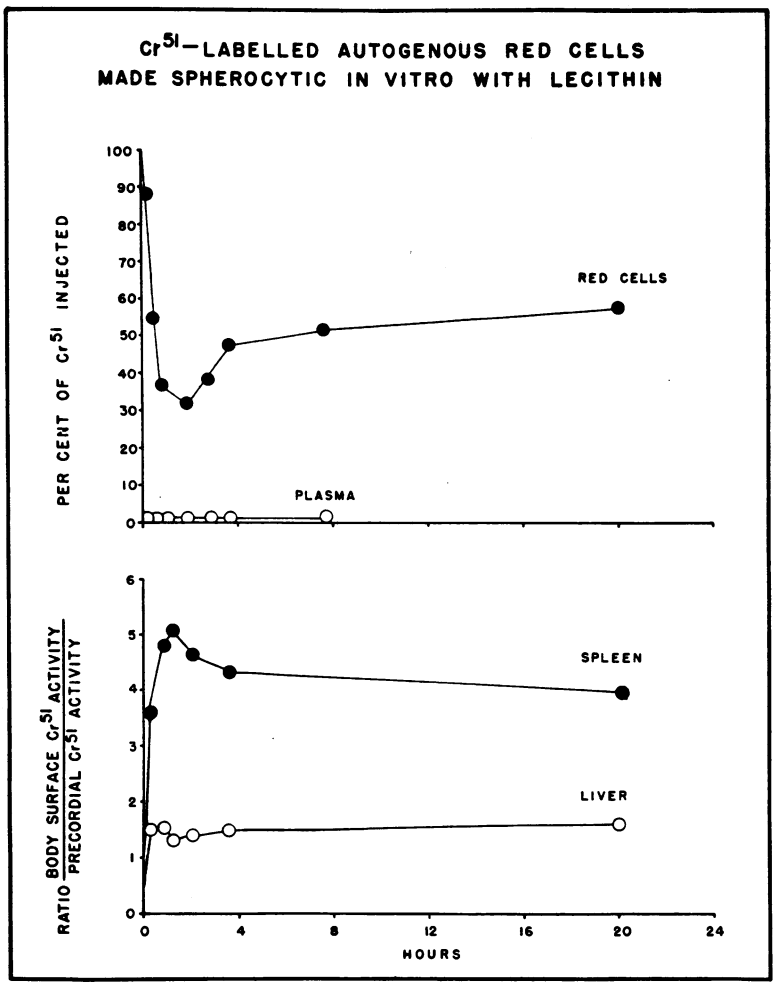

FIG. 10. SPLENIC SEQUESTRATION OF LECITHINIZED SPHEROCYTES

The rate of sequestration of these spherocytes resembled that of red cells coated with incomplete antibodies (2). However, the apparent "escape" from the spleen of a portion of these red cells and the complete absence of hemoglobinemia were never observed after comparable injections of anti-D sensitized red cells. Note that many of the cells were detained in the spleen for three or four hours, and some for as long as 22 hours. Thereafter (not shown) red cell Cr^{51} levels declined slowly in a normal fashion. Although in this experiment approximately 7 or 8 ml. of red cells was sequestered in the spleen, there was no accompanying leukopenia or febrile reaction.

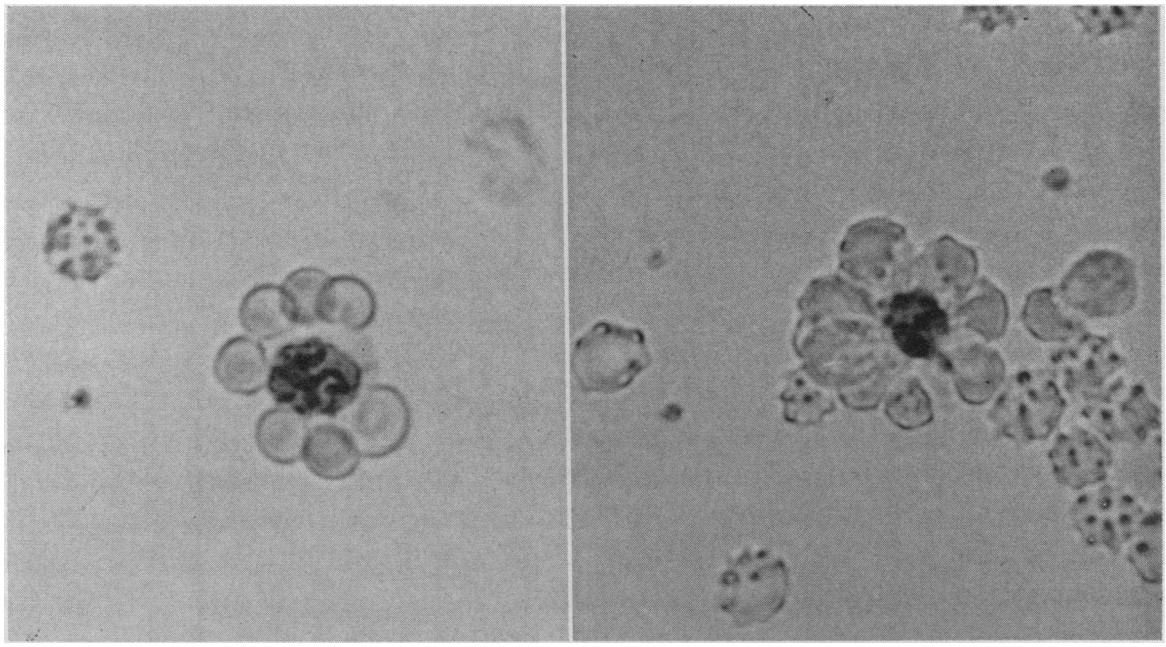

FIG. 11. ROSETTE-TYPE AGGLUTINATES INVOLVING ANTI-D SENSITIZED RED CELLS AND GRANULOCYTES FROM A D-SENSITIZED SUBJECT

Following incubation at 37° C. for 30 minutes of type D red cells suspended in resin-decalcified incomplete anti-D plasma to which freshly-obtained leukocytes from a D-sensitized donor had been added, there was no red cell agglutination. However, red cell clumping around leukocytes (usually granulocytes) was apparent as shown. The preparation was diluted with saline (thus causing some red cell crenation), stained supravitally with neutral red, and photographed under a cover slip. Note the smooth, spherocytic contour of the adherent red cells, particularly on the left, the "invasive" appearance of the granulocyte processes, particularly on the right, and the lack of involvement of platelets.

passage through a cation-exchange resin column, and serum from defibrinated blood,[8] respectively.

In such systems containing type A red cells and type O plasma and leukocytes, immediate red cell agglutination was microscopically visible. At the periphery of many of the clumps one or more leukocytes (invariably granulocytes or monocytes) were seen to be adherent. Red cells contiguous to these leukocytes underwent sphering within a few minutes, and shortly thereafter erythrophagocytosis frequently became evident. Platelets were also seen to adhere to these leukocytes, but no more than to "free" leukocytes. Apart from these mixed agglutinates, clumping of leukocytes or of platelets did not occur to a greater extent than in control mixtures in which red cells were suspended in compatible plasma. The total numbers of leukocytes and of platelets were unchanged during the period of observation.

In systems involving type D red cells and plasma (containing incomplete anti-D) and leukocytes from D-sensitized subjects, no red cell agglutination occurred. Within a few minutes of admixture, however, the antibody-sensitized red cells were seen to cluster around individual leukocytes, often producing rosette-patterns (Figure 11). These red cells also became spherocytic. Erythrophagocytosis occurred to only a small extent, and then only after an hour or two. Occasionally two or more leukocytes were involved in mixed agglutinates. Platelets were not involved in these mixed clumps, and there was no evidence of pure

[8] Whereas defibrinated blood is to be preferred to plasma containing anticoagulants when the action of complement is under study, it is less satisfactory for harvesting leukocytes. We have observed that in the course of defibrinating whole blood with glass beads, a sharp reduction of leukocyte numbers occurs within a minute of the onset of fibrin-deposition. This reduction in leukocytes results almost entirely from adherence of granulocytes to the clot.

leukocyte or platelet agglutination. The total leukocyte and platelet counts were unchanged. When mixtures were made of type D red cells, plasma containing anti-D, and leukocytes from unsensitized subjects, adherence of red cells to leukocytes occurred more slowly and to a lesser extent; in such suspensions mixed agglutination became prominent only when the cells were lightly centrifuged together.

In an effort to circumvent the possible artificialities of *in vitro* preparations, several observations were carried out within the vascular bed of the tourniquet-obstructed forearms of normal human subjects. In these studies the subjects were given 50 mg. of heparin intravenously, and Cournand needles were placed in the antecubital veins of each arm. A blood pressure cuff was then inflated around one arm at a pressure midway between systolic and diastolic blood pressures, and immediately thereafter a small volume of Cr^{51}-labeled red cells was injected into the brachial artery just below the inflated cuff. Small specimens of venous blood were withdrawn into heparin at frequent intervals through each Cournand needle before and after deflation of the cuff, and were examined for radioactivity, for leukocyte levels, and, in blood suspended in warm saline, for autoagglutination.

In each of two observations approximately 2 ml. of Cr^{51}-labeled type A-incompatible red cells was injected intra-arterially into the tourniquet-obstructed forearm. As portrayed in Figure 12, maximal numbers of labeled red cells soon appeared in the local venous samples, but such radioactivity then slowly fell, presumably as a result of the escape of venous blood past the cuff and of venous flow through the humerus. In the first venous samples containing labeled red cells, red cell agglutinates were visible. Often leukocytes were attached to the periphery or occasionally

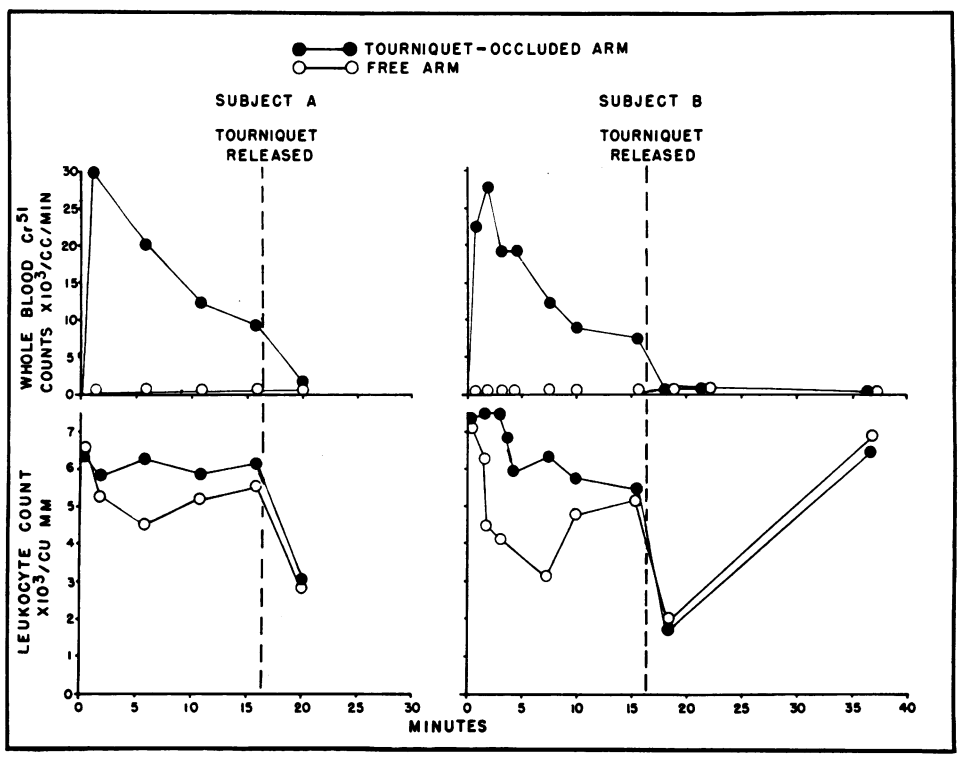

FIG. 12. THE EFFECT ON LEUKOCYTE LEVELS OF Cr^{51}-LABELED INCOMPATIBLE RED CELLS INJECTED INTRA-ARTERIALLY INTO THE TOURNIQUET-OCCLUDED FOREARMS OF NORMAL SUBJECTS

Results are of observations made in two subjects. Note that despite active local red cell agglutination and hemolysis, leukolysis in the "tourniquet-isolated" arm did not occur. However, the systemic effects of immune red cell agglutination are apparent in the systemic leukopenia that promptly followed the release of the tourniquet.

were present in the center of these agglutinates; and, as *in vitro,* the red cells adjacent to these leukocytes appeared to be spherocytes. Nevertheless, there was little or no reduction in leukocyte levels in the venous blood samples from the "obstructed" arms, while venous blood in the opposite, "free" arm, which registered the presence of only traces of Cr^{51}-labeled red cells, showed transient leukopenia, presumably as an effect of those injected red cells which escaped from the obstructed arm. Meanwhile, in venous samples from the obstructed arm progressive hemoconcentration developed and the plasma hemoglobin level rose to over 100 mg. per cent in each observation, although no hemoglobin was detected in plasma from the opposite forearm. In each observation, on deflation of the cuff the Cr^{51} activity of the red cells and the leukocyte level both abruptly fell in venous blood from the obstructed arm. Subsequent body surface scanning revealed a heavy accumulation of Cr^{51} in the subjects' livers, and to a lesser extent in their spleens, but no retention of Cr^{51} activity was detectible in the subjects' previously obstructed forearms.

In a single comparable observation, in which 2 ml. of Cr^{51}-labeled, anti-D sensitized autogenous red cells was injected intra-arterially into the obstructed forearm of a normal subject, there was no effect on leukocyte levels in either arm during a 15 minute period of venous stagnation, and no mixed agglutinates were seen in local venous samples.

Observations with incompatible red cells injected intradermally

In order to examine the possibility that cells other than leukocytes may be injured by the reactions between red cells and their antibodies, compatible and incompatible red cells were injected intradermally into several subjects. Generally 0.1 ml. of a 50 per cent saline suspension of washed red cells was injected intradermally into the flexor surface of the forearm: test cells at one site and autogenous or compatible cells at another.

TABLE III

Relation between the dermal response to ABO-incompatible red cells and isoantibody titers

Subject	Injected red cell type	Subject's red cell type	Subject's isoantibody titer*		Skin response (wheal and erythema)
			Hemolysin	Agglutinin	
			Positive skin responses		
JA	B	A	4	128	4+
WI	A	O	4	128	4+
PU	A	O	4	32	4+
VA	B	A	1	64	3+
OR	A	B	1	32	2+
WA	A	O	1	64	2+
WA	B	O	Trace	64	1+
WL	A	O	Trace	64	2+
DA	A	O	0	128	1+
			Negative skin responses		
WI	B	O	0	32	0
PU	B	O	0	32	0
WL	B	O	0	16	0
DA	B	O	0	8	0
O'L	A	O	0	32	0
O'L	B	O	0	128	0
ME	B	O	0	64	0
HO	B	A	0	8	0
GO	A	O	0	32	0
GO	B	O	0	8	0
VI	A	O	0	64	0
VI	B	O	0	16	0
MC	A	O	0	64	0
MC	B	O	0	8	0
PH	A	O	0	64	0
PH	B	O	0	64	0

* Measured by incubating equal volumes of various dilutions of fresh serum and 5 per cent washed red cells in saline for one hour at 37° C. The titer is expressed as a reciprocal of the minimally effective serum concentration.

Most subjects injected with 0.1 ml. of ABO-incompatible red cells manifested no immediate or delayed skin reactions and the progress of visible pigment breakdown proceeded at the same rate as in the sites of injection of autogenous red cells. However, approximately one-third of normal subjects developed a wheal at the site of injection of incompatible red cells (Table III and Figure 13). Generally wheal formation became apparent within 2 to 5 minutes of injection, the swelling was maximal in 10 to 20 minutes, and a diminution of swelling was noticeable in about one hour. A surrounding erythema and local venous constriction were usually evident simultaneously during the onset of wheal formation. Two subjects noted transient itching during wheal formation, and occasionally during the ensuing 24 to 48 hours. As noted in Table III, those subjects who developed urticaria at the site of injection of incompatible red cells usually possessed hemolysins for those cells. None of the subjects with isohemolysins demonstrable by conventional techniques *in vitro* failed to develop an acute wheal in response to intradermal incompatible cells. Only one subject not manifesting isohemolysins *in vitro* exhibited dermal sensitivity to incompatible red cells. Ten type O subjects were injected intradermally at different sites with type A and with type B cells. In the three subjects with isohemolysins for type A red cells, but not for type B cells, there were positive skin reactions to type A cells only.

Two type A subjects who were injected intradermally with 0.1 ml. of type O serum which hemloyzed type A cells showed small, questionable wheals and no erythema at the injection sites. However, when the same type O serum was mixed with the subjects' type A cells *in vitro,* and 0.1 ml. of the mixture was injected, a very strong wheal-and-erythema response occurred (Figure 13).

The injection of 0.1 ml. of a 50 per cent suspension in saline of type D red cells into two otherwise normal D-negative subjects sensitized to the D antigen caused no immediate or delayed skin reactions. Similarly the intradermal injection into two subjects of type D red cells sensitized *in vitro* with anti-D serum produced no skin reactions. That wheal formation was not caused by the injection of red cell agglutinates *per se* was demonstrated by injecting 0.1 ml. of washed autogenous red cells which had been strongly agglutinated by ferric chloride. There was no dermal reaction.

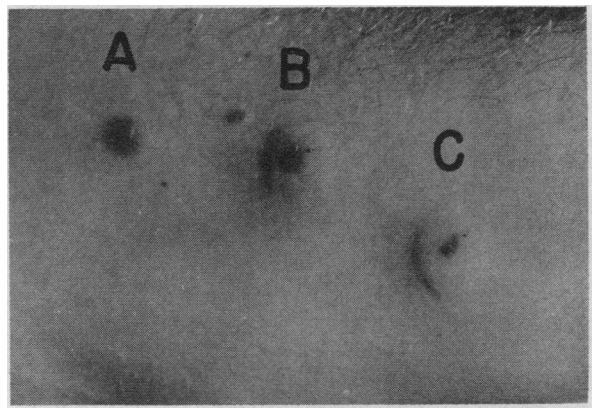

FIG. 13. IMMEDIATE SKIN REACTIONS PROVOKED BY ISOHEMOLYSIS

Three injections were given intradermally within a total period of 4 or 5 minutes and this photograph was taken 40 minutes later. The injection into this type A subject of 0.1 ml. of a 50 per cent suspension of type O red cells in saline caused no reaction (site A). The injection of a comparable preparation of type B red cells, however, caused an immediate wheal and erythema response (site B). A similar, even more intense wheal and erythema response followed the injection of site C of a mixture of autogenous type A red cells and type O serum containing isohemolysins. Injections of autogenous serum alone (not shown) left little or no swelling. Injections of serum containing isohemolysins against the subject's red cells (not shown) provoked a slight wheal without erythema.

DISCUSSION

Leukopenia during immune hemolysis

The observations presented indicate that leukopenia is a characteristic feature of immune hemolysis *in vivo,* whether this hemolysis arises from hemolysins or from complete or incomplete agglutinins. The leukopenia, initially at least, reflects a fall in granulocyte and monocyte levels. The leukopenic response is generally proportional to the maturity of these cells, thus being striking with respect to adult neutrophils, moderate with band forms, and absent with metamyelocytes.

The leukopenia is clearly not a consequence of the direct action of antibodies on leukocytes since it is most readily produced in subjects by the injection of washed incompatible red cells. It is also clear that the leukopenia is not simply the result of antigen-antibody interaction, since the intravenous

injection of anti-A serum into type A recipients and of anti-D serum into type D recipients caused little or no leukopenia, unless actual hemolysis was produced. That leukopenia appears to reflect some feature of the immune lysis of red cells rather than the mere coating of the cell with antibodies or the hemolysis *per se* is indicated by the following observations: *1*) Leukopenia occurred slowly with the slowly-sequestered and -hemolyzed anti-D sensitized red cells, and rapidly with the rapidly-sequestered and -hemolyzed ABO-incompatible cells. *2*) When antiserum was injected intravenously in amounts insufficient to cause hemolysis *in vivo* no leukopenia ensued, whereas when the same volume of antiserum was mixed with the recipients' incompatible red cells prior to injection, resulting in the hemolysis of these cells *in vivo,* leukopenia occurred. *3*) The injection of whole red cell hemolysates prepared by freezing and thawing or of osmotically released hemoglobin had no effect on circulating leukocytes nor did the brisk sequestration and destruction of red cells *in vivo* by several nonimmunologic mechanisms, including preliminary alteration of the injected red cells by prolonged incubation or by exposure to lecithin or acetylphenylhydrazine. It is also evident that a response by leukocytes to foreign red cells as such does not underlie the leukopenia, since anti-D coated autogenous red cells were active in this respect; furthermore, a patient with acquired hypogammaglobulinemia, who was injected with ABO-incompatible red cells and who failed to destroy these cells rapidly (*2*) showed no leukopenic response to the incompatible cells.

The finding that leukopenia followed the injection of soluble A substance into type O individuals, but not into type A individuals, indicates, nevertheless, that leukopenia may result from antigen-antibody interaction when these are in a noncellular suspension or solution. Indeed it is well established that the injection into sensitized animals of noncellular antigens provokes leukopenia (20–23) and often thrombocytopenia (24) much as does the injection of bacterial extracts ("pyrogens" or "endotoxins") into normal animals, including man (25–30).

The observations *in vitro,* as well as in the tourniquet-obstructed forearm, indicate that leukocytes, particularly granulocytes and monocytes, tend to adhere strongly to antibody-coated red cells. In systems containing ABO-incompatible serum and red cells our observations are similar to those on the action of complement-fixing dog and human isoantibodies by Bakemeier and Swisher (12) who described the adherence *in vitro* of leukocytes to red cell agglutinates in the absence of hemolysis, and the formation of heavy mixed agglutinates of red cells and leukocytes when hemolysis was present. Butler (31) has described clearly the evolution *in vitro* of mixed agglutinates due to ABO-antibody-coated red cells, showing that the leukocytes were attached in common to the red cells rather than to each other. Neither *in vitro* nor *in vivo* was there evidence of direct leukolysis or of pure leukoagglutination. In our hands a tendency for leukocytes to adhere to antibody-coated red cells was also evident in the absence of complement fixation or of red cell agglutination. Thus, anti-D sensitized red cells clustered about, and adhered to, leukocytes (particularly those of D-sensitized subjects) to form rosettes, as in Figure 11. In general the tendency for leukocytes to participate in mixed agglutinates varied directly with the speed with which the involved antibody caused hemolysis *in vivo,* and the speed and extent with which erythrophagocytosis occurred *in vitro.*

Although a tendency exists for contiguous leukocytes to adhere to antibody-coated red cells, it is unlikely that the leukopenia observed *in vivo* results directly from a filtration from the circulation by certain capillary beds of red cell-leukocyte clumps or of erythrophagocytic leukocytes. The improbability of such a mechanism may be inferred from the facts that leukopenia follows the injection of soluble A substance and that leukocytes may be sequestered within a few minutes of the injection of incompatible red cells in a ratio as great as 15 leukocytes to 1 red cell (Table I). It is more likely that the leukopenia results from a mechanism common to all intravascular antigen-antibody reactions, particulate or nonparticulate, and is akin to the physiologic response to bacterial endotoxins.

The conclusion most consistent with the observations summarized above is that the reaction of antibody with antigen on the red cell surface causes a change in the surface of nearby leukocytes, and that this leads to mutual adhesion and

often to actual phagocytosis. However, striking leukopenia occurs only when hemolysis of the sensitized red cells, either by plasma or by tissue factors, releases (in comparative profusion) the antigen-antibody complexes into the bloodstream. This presumably is what occurs without the vehicle of the red cells when nonparticulate antigens or bacterial endotoxins are injected intravenously.

The general nature of the change in the leukocyte surfaces which attends the interaction between antigens and antibodies, and, as well, the action of bacterial endotoxins, is an increase in the "stickiness" of leukocytes, thus disposing them to adhere to one another and probably to vascular endothelium everywhere (*22*, 32–34). Presumably filtration and adhesion of such leukocytes would be most prominent in those organs with large or effective filtering beds, namely the lungs, liver and spleen. Indeed, there have been numerous reports of cramming of the pulmonary vasculature with leukocytes during anaphylaxis (*26*, *27*, 35).

The leukocytes most affected in this manner by immune reactions are the granulocytes and monocytes. In the present study eosinophils and probably basophils also were so affected, whereas lymphocytes were conspicuously spared. The lymphopenia which occurred about two hours after the injection of red cells, particularly when anti-D sensitization was involved, in all likelihood reflects an adrenocortical effect. Our failure to observe the thrombocytopenia after the injection of incompatible red cells described by others as a result of incompatible transfusions (11, 36) and said to be associated with platelet-red cell mixed agglutinates (11, 36), may reflect the smaller quantity of incompatible red cells injected by us and the smaller amount of complement fixation involved. Indeed, thrombocytopenia is known to accompany anaphylaxis in certain instances (24), and possibly the susceptibility of platelets to injury by antigen-antibody reactions is analogous to, but less than, that of leukocytes.

The actual fate of the leukocytes which disappeared from the circulation in this study, as in similar studies in general, is not entirely clear. It is possible that many of these cells were only transiently sequestered or that they temporarily emigrated from the vasculature (32) and returned within a few hours to the general circulation. However, it is clear that the initial recovery of leukocyte levels was due not to the return of the sequestered leukocytes, but in large part, at least, to the appearance of young cells including bands, metamyelocytes, myelocytes, and some immature monocytes. This outpouring of young leukocytes was evident within less than an hour after the injection of ABO-incompatible red cells, whereas in dogs subjected to mechanical leukophoresis, Craddock, Perry, and Lawrence (37) have found that a delay of about two hours generally precedes the initial rise in leukocyte levels. This delay is believed to reflect a "priming" of the leukocyte-depleted vasculature. Their finding that the immature leukocytes which appear during the leukocytosis are derived from the marrow pool, whether the preceding leukopenia was the result of leukophoresis (37) or of the injection of typhoid vaccine (38), almost certainly applies here.

The possible physiological utility of the extraordinary leukocyte responses to immune hemolysis is conjectural. Evidence for the participation of circulating leukocytes in the phagocytic destruction of antibody-sensitized red cells *in vivo* is largely limited to immune systems involving complement fixation. In its absence there is usually meager evidence of erythrophagocytosis *in vivo;* and erythrophagocytosis *in vitro* of red cells sensitized with anti-D serum or of red cells from most patients with acquired hemolytic anemia is comparatively slow to appear and limited in extent. Nevertheless, the studies *in vitro* of Zinkham and Diamond (39), Butler (31), and Bakemeier and Swisher (12), as well as those described here, indicate that antibody-sensitized red cells, and the "sensitized" red cells of acquired hemolytic anemia, whether or not complement is involved, tend in various degrees to cling to granulocytes and monocytes. This phenomenon consists initially of red cell adherence to contiguous leukocytes, associated with apparent "sphering" of the involved red cells; actual phagocytosis of the trapped red cell may then supervene, particularly if complement is fixed. Of course, for sensitized red cells to be destroyed by fixed or circulating leukocytes it may not be essential that actual erythrophagocytosis occurs. Turner (40) has reported that leukocytes may act as cofactors in the lysis by cobra venom of the red cells of certain animal species by providing a source of lecithin which is rendered hemolytic by the venom

lecithinase. Nevertheless, we did not succeed in making analogous observations by incubating together various combinations of leukocytes, lecithin, fresh serum and normal or anti-D sensitized red cells. In general, however, it seems plausible that, by virtue of the phenomena described above, the circulating leukocytes may aid the fixed macrophages of the reticuloendothelial system in destroying trapped, antibody-sensitized red cells. Jointly their function may be considered that of cleaning the filter beds of the spleen, liver and lungs that are mechanically responsible for the initial sequestration of the red cells.

That anti-D sensitized red cells adherent to leukocyte surfaces appear spherocytic is evident in Figure 11. Such spherocytosis is also apparent in photographs of the fixed smears of Zinkham and Diamond (39), which show sensitized red cells of acquired hemolytic anemia patients clustered around leukocytes prior to erythrophagocytosis. A mysterious feature of immune hemolysis has been that the action of antibodies on red cells *in vivo* leads to spherocytosis, whereas this is not so when such antibodies are incubated with red cells *in vitro* (1). Similarly spherocytosis is a common feature of the peripheral blood of patients with acquired hemolytic anemia (41), whereas incubation of normal red cells with the serum of these patients does not cause spherocytosis even when sensitization or agglutination is provoked. It is tempting to conclude that the spherocytosis observed *in vivo* during immune hemolysis and in patients with acquired hemolytic anemia is a consequence of the surface effects of leukocytes and of tissue macrophages upon antibody-coated red cells. Thus far our efforts to demonstrate *in vitro* changes in the osmotic fragility of antibody-sensitized red cells in the presence of leukocyte suspensions have perhaps been frustrated by the comparatively small number of red cells involved in the mixed agglutinates which develop.

Fever during immune hemolysis

Fever and chills often accompany severe hemolytic episodes, such as attend transfusions of incompatible homologous or heterologous blood. However, the mechanism of this pyrogenic response has been obscure. In their search for tissue pyrogens, Bennett and Beeson (42) found that red cells hemolyzed *in vitro* by various nonimmunologic procedures failed to provoke fever on injection into normal rabbits, whereas the injection of dog serum containing a high titer of agglutinins against rabbit red cells, in distinction to dog serum inactive against rabbit red cells, did produce a febrile response. That the presence of a foreign protein or tissue need not underlie the fever of such hemolysis, however, is evident, in that patients with paroxysmal cold hemoglobinuria develop comparable pyrogenic responses following hemolytic paroxysms induced by cooling of an extremity (7, 8).

The findings reported here as to the effects of immune hemolysis in man on body temperature may be summarized as follows: *1*) Hemolysis by isoantibodies in man was generally attended by a febrile response. *2*) Hemolysis of comparable degree by nonimmunologic mechanisms did not cause fever. *3*) The slow destruction *in vivo* of red cells by incomplete antibodies (*i.e.*, anti-D) produced decidedly more striking febrile responses than did the rapid destruction of comparable amounts of red cells by complete agglutinins or hemolysins (*i.e.*, anti-A and anti-B), although the latter agents induced much more hemoglobinemia. *4*) A febrile response accompanied immune hemolysis regardless of whether the interacting red cells or antibodies, or neither, were native to the subject. *5*) The pyrogenic response was invariably accompanied by leukopenia, and the onset of fever followed approximately one hour after the injection of antigenic cells or antibody. *6*) Finally, as with the leukopenia, fever did not occur when red cells and antibody interacted *in vivo* unless red cell sequestration or hemolysis was also produced.

An interesting association between the rate of hemolysis and the occurrence of fever emerged from the studies summarized above. The rapid sequestration of red cells by anti-A or anti-B was associated with a rapid, transient leukopenia and with only slight fever. The slower sequestration of red cells by anti-D was accompanied by a more slowly developing and more prolonged leukopenia and with marked febrile responses.[9] These two

[9] It is presumed that the rather striking differences observed in these studies between the effects of these two immune systems are in part a function of the number of red cells involved and that these differences may be obscured when the mechanisms of immune destruction are saturated by the comparatively large amounts of red cells generally involved in transfusions of incompatible blood.

patterns of response, respectively, to ABO-incompatibility and to D-incompatibility resemble those which occur when bacterial pyrogens are injected into "tolerant" rabbits and into normal rabbits, respectively. Tolerance to injections of pyrogens in rabbits is characterized by an accelerated removal of the pyrogenic material from the bloodstream (43–45) and thereafter a diminished febrile response. This pattern may be likened to that reported here with ABO-incompatibility. In contrast, pyrogens injected into normal rabbits are removed comparatively slowly from the blood stream and fever is pronounced (43–45), as is the case with the slow immune destruction of type D red cells. It may be postulated that the slow removal of bacterial products or of antigenic materials is attended by more widespread tissue alterations than is possible when abrupt removal of the material occurs. It is alternatively conceivable that such slow rates of particle-removal reflect simply the fact that the spleen is providing the chief site of particle-sequestration, and that in the spleen special conditions may exist favoring the alteration of crucial cell types (*i.e.*, leukocytes, platelets, reticuloendothelial cells, lymphatic tissue). In support of such a possibility was the observation (not reported above) that the injection of Cr^{51}-labeled, anti-D sensitized red cells into two splenectomized subjects was followed by a comparatively slow sequestration of the sensitized red cells by the subjects' livers (2). The usual strong febrile response one hour after injection did not appear but, rather, a slight prolonged temperature elevation of less than 1° F. It is conceivable in view of such considerations that the severity of the febrile paroxysms in malaria depends upon the splenic sequestration of parasitized red cells. That the spleen, possibly for similar reasons, plays a unique role in the formation of antibodies to particulate antigens is indicated by the fact that splenectomized human (46) or animal (47) subjects form antibodies in response to intravenously injected heterologous red cells much less readily than do normal subjects, whereas the antibody response of splenectomized animals to particulate antigens when injected subcutaneously is unimpaired (47). Also it seems likely that the natural (48) or experimental (49) sensitization of D-negative subjects occurs less readily with ABO-incompatible type D red cells than with ABO-compatible type D red cells because ABO-incompatible cells are abruptly removed from the circulation after injection, largely in the liver, and are afforded comparatively little chance for contact with specially-disposed lymphatic tissue, such as that of the spleen.

There is still uncertainty as to whether the febrile response to exogenous bacterial pyrogens is ordinarily mediated through the release of an endogenous pyrogen (42, 44), or results from a direct effect of exogenous pyrogen on the central nervous system (50), or both. However, there is much evidence that endogenous pyrogens exist in granulocytes (42), and that in man the direct interaction of bacterial pyrogens and leukocytes *in vitro* leads to the liberation of rapidly-acting pyrogenic material, as shown by Cranston, Goodale, Snell, and Wendt (51, 52). It is therefore of interest that when anti-D sensitized red cells were incubated *in vitro* with an even larger quantity of leukocytes than was employed by Cranston and co-workers, there was no hastening of the pyrogenic effect. Actual red cell lysis by the immune mechanism appears to be required for the febrile response as well as for the leukopenic response. The reinjection of soluble bacterial (23) and nonbacterial (53) antigens has been shown to produce fever as well as leukopenia (20–23). Therefore the association of both fever and leukopenia with actual lysis by immune mechanisms suggests that both reactions are caused by the release into the blood stream of antigen-antibody complexes from hemolyzing red cells.

Dermal responses to immune hemolysis

With one exception, incompatible red cells injected intradermally caused wheal and erythema reactions only when some degree of lysis of the red cells by the subject's serum was demonstrable *in vitro*. Likewise, intradermal injection of autogenous red cells after incubation with hemolytic (anti-A) sera *in vitro* caused similar responses. These observations suggest that immune lysis by complement fixation provoked the dermal reaction. The wheals resembled those produced by the injection of histamine or of allergens into sensitive subjects. The pronounced constriction of nearby veins which appeared after the injection of ABO-incompatible red cells into appropriate subjects also suggested the action of 5-hydroxytryptamine

(serotonin) (54). Serotonin has been shown to cause edema in animals and to account for much of the edema provoked by foreign proteins (55). Furthermore, it has been shown that serotonin (56), as well as histamine (57), is released when antigen-antibody reactions occur in the presence of blood cells *in vitro,* and that in the case of serotonin, platelets appear to be the cellular source and complement is apparently involved (56). In rabbits, anaphylaxis is associated with immediate rises in plasma levels of serotonin and histamine, and concomitant falls have been recorded in the levels of these substances in blood cells (58).

In order to determine whether serotonin produced dermal effects resembling those of red cells undergoing immune lysis, two normal subjects were injected intradermally with 0.1 ml. volumes of saline containing various amounts of serotonin. In these two subjects 0.1 μg. of serotonin creatinine sulfate [10] produced a doubtful, and a small area, respectively, of erythema at the injection sites. In each subject, 1 μg. caused an erythematous area (30 to 35 mm. in diameter) to appear at the injection site, neighboring superficial veins became constricted, and a small central wheal appeared. The response reached a maximum in 10 to 12 minutes and persisted for over an hour. The injection of 10 μg. of serotonin intradermally provoked a similar response, except that erythema and venous constriction were evident over a wider area, and the effect lasted two hours. This response to intradermal serotonin was similar to that produced by intradermal antibody-lysed red cells, except that wheal formation was more prominent in the latter. The intravenous injection of 1 mg. of serotonin into a normal subject had no effect on leukocyte levels.

Taken together, the following observations strongly suggest that certain isoantibodies may, in causing hemolysis, provoke the release of histamine, serotonin and possibly other related substances from leukocytes and platelets: *1*) the finding of others (56–58) that antigen-antibody reactions with the probable involvement of complement cause a release of histamine and of serotonin from leukocytes, platelets and certain other cells; *2*) the observation of others (11) that red cell lysis *in vivo* by complement-fixing antibodies injures platelets as well as leukocytes; *3*) the observation made here that red cell hemolysates produced by complement-fixing antibodies provoke dermal reactions resembling those produced by histamine and serotonin; and finally, *4*) the observation made here that intravenous injections of small amounts of type A cells or of soluble A substance into type O recipients may cause an acute syndrome of facial flushing, dyspnea and hyperactive peristalsis. Similarly, during hemolysis by the complement-fixing autoantibodies of paroxysmal cold hemoglobinuria, urticaria often appears. Although attributed to a specific "dermolysin" (59) this urticaria is partly suppressed by antihistamines (60) and may reasonably be attributed to the sequence of events postulated to occur during hemolysis by isohemolysins.

That immune destruction of red cells, like other immune processes, may indirectly evoke certain tissue reactions is apparent in the ability of intravenously injected antibody-sensitized red cells to provoke the Shwartzman phenomenon in prepared skin sites of rabbits (61). It is of further interest that bacterial endotoxins, which prepare skin for the local Shwartzman reaction [which, in turn, involves the local formation of thrombi of platelets and leukocytes (22, 23, 33)], induce a state of remarkable sensitivity to the action of serotonin (62). Davidsohn and his associates (63) have described suppurative skin lesions in rabbits at the site of injection of anti-rabbit-red cell hemolytic immune serum. The authors postulated that this effect reflected an antigenic similarity between red cells and skin; and, indeed, it is known that human epidermis may contain A and B antigens (64, 65). However, the skin reactions described here were minimal after direct injections of antiserum, and required for full development the presence of red cells. Thus, indirect, rather than direct, toxicity to skin was evidently involved.

A scheme summarizing the observed and postulated effects of immune hemolysis on the host is presented in Figure 14. Although certain striking distinctions have been emphasized in this report between the effects of hemolysis by anti-D and by anti-A or anti-B, it is believed that these distinctions are in part quantitative. Therefore, the two hemolytic systems are not differentiated in

[10] Kindly supplied by the Research Laboratories of the Upjohn Company, Kalamazoo, Mich.

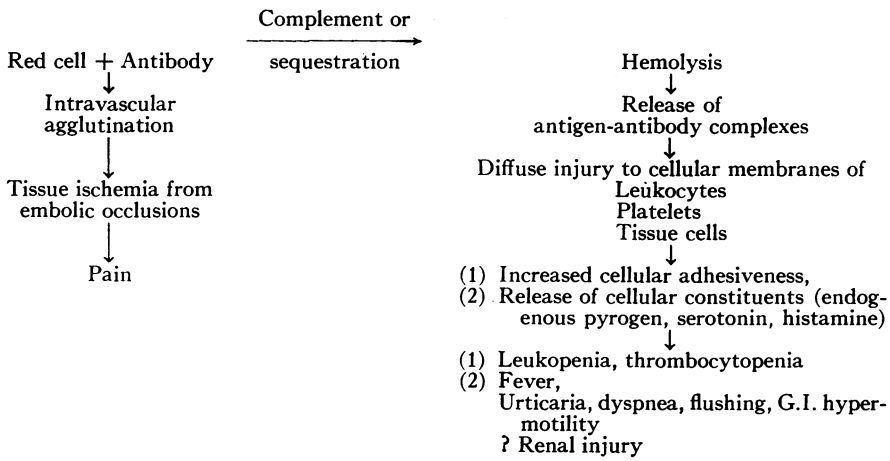

FIG. 14. PROPOSED MECHANISMS UNDERLYING SYSTEMIC REACTIONS TO HEMOLYSIS

Figure 14. However, it may be recalled in examining this scheme that the systemic effects of hemolysis by the ABO system primarily include certain immediate reactions, believed to reflect the effects of intravascular agglutination and of release of histamine and serotonin, whereas hemolysis by anti-D may be likened in its effects to substances such as tuberculin which cause delayed sensitivity, in which acute reactions are absent but in which fever is a striking feature. Finally, there is much evidence to support the contention that, just as anaphylaxis may arise from the release of histamine and serotonin from certain cells injured by the antigen-antibody reaction, fever following immune hemolysis may represent the action of endogenous pyrogen released from granulocytes (42, 44, 51, 52) similarly injured.

As noted earlier, it may be difficult, in considering acute hemolytic episodes, to ascertain to what extent anoxia, hemoglobinemia, embolization and shock may contribute to the injury sustained by the patient. Almost certainly, however, anaphylactic and pyrogenic phenomena contribute heavily to the acute damage,[11] and quite possibly to the more slowly apparent renal damage. It is doubtful that the renal shutdown observed in transfusion reactions or in paroxysms of cold hemoglobinuria (67) is caused by hemoglobinuria *per se,* unless this is preceded by functional tubular abnormalities (68). The danger of embolic occlusions in the kidney, as well as elsewhere, undoubtedly exists when hemolysis involves agglutinating antibodies, and focal cortical ischemia may be a prominent finding in the kidneys of patients dying of acute renal failure following mismatched transfusions (69). However, experimental efforts to demonstrate embolic intrarenal red cell agglutinates in animals during heterologous transfusion reactions have not been successful (70). It seems likely that the pyrogenic response, particularly when prolonged, may contribute to the pathogenesis of renal tubular injury by producing hypotension (71) and by direct derangement of renal function (72).

SUMMARY

Destruction of red cells by isoantibodies in normal human subjects was accompanied by a profound leukopenia, affecting granulocytes and monocytes. The abrupt, primarily hepatic, sequestration of ABO-incompatible red cells produced an abrupt leukopenia, while the slower, mainly splenic, sequestration of red cells coated with incomplete (anti-D) antibodies was accom-

[11] Accordingly, when symptoms of dyspnea, flushing and gastrointestinal disturbances occur very shortly after the onset of acute hemolysis, as by transfusion of incompatible blood, the use of parenteral agents which inhibit the action of histamine and serotonin (*e.g.,* chlorpromazine) may prove useful. In addition, antipyretics are certainly advisable in order to subdue the pyrogenic response, which generally occurs approximately an hour after the onset of hemolysis. It is interesting to note that, in the studies reported above, cortisone had no effect on the rate of blood destruction or on the leukopenia, but acted simply as an antipyretic (presumably on the central nervous system), as has been described in animals exposed to bacterial pyrogens (66).

panied by a synchronous, slowly developing leukopenia. In each instance there followed a leukocytosis, initially composed of immature granulocytes.

Leukopenia did not appear during hemolysis *in vitro* by nonimmunologic mechanisms involving either hepatic or splenic filtration of red cells. Although injections of soluble A substance into subjects whose sera contained anti-A did provoke leukopenia, the reaction of isoantibodies with red cells affected leukocyte levels only when actual red cell sequestration or lysis transpired.

Observations *in vitro* revealed that red cells coated with anti-D antibodies strongly adhered to leukocytes, particularly those of D-sensitized subjects. Similarly, mixed agglutinates containing leukocytes (regardless of blood type) and of red cells sensitized by ABO-incompatible plasma were encountered, confirming observations of others. In either instance, antibody-coated red cells adherent to leukocytes were transformed into spherocytes. Erythrophagocytosis was prominent only when red cells underwent direct hemolysis by complement. Despite the striking interactions between leukocytes and sensitized red cells observed *in vitro* and in the venous tourniquet-obstructed arms of normal subjects, there was no direct leukolysis; furthermore, the leukopenic effect probably did not result simply from the sequestration of mixed agglutinates.

The mechanism of the leukopenia (and possibly of the thrombocytopenia described by others) accompanying acute immune hemolysis, is believed to be analogous to that known to occur during certain other antigen-antibody reactions and following injections of bacterial endotoxins. Here, too, the injurious action on leukocytes (and platelets) of antigen-antibody complexes released from the hemolyzing red cell presumably causes the leukocytes to adhere to endothelial surfaces, particularly in organs with large capillary beds.

Febrile reactions, like leukopenia, accompanied hemolysis *in vivo* by immune, but not by nonimmune, mechanisms. Under the experimental conditions, marked fever occurred only during hemolysis by incomplete (anti-D) antibodies, and this occurred regardless of whether the interacting red cells or antibodies, or neither, were native to the subject. These reactions were characteristic of pyrogenic responses in general in their delayed appearance and were also analogous to reactions to bacterial pyrogens in that slow removal of the "pyrogen" from the blood stream provoked the more severe febrile responses.

The intracutaneous injection of red cells into ABO-incompatible subjects manifesting hemolysins for these red cells *in vitro* provoked immediate, transient, local skin reactions consisting of wheal formation, erythema and venous constriction. Such reactions also followed intracutaneous injections of autogenous red cells hemolyzed *in vitro* beforehand by isoantibodies. It is believed that these dermal effects represent the release of histamine, serotonin and related compounds from leukocytes and platelets injured by immune hemolysis.

On the basis of these findings it is suggested that many of the systemic effects of immune hemolysis (leukopenia, thrombocytopenia, chills and fever, dyspnea, flushing, urticaria, gastrointestinal disturbances, and possibly renal injury) reflect in part the injurious effects of the released antigen-antibody complexes on leukocytes and platelets. This injury is believed to consist of a change in the surface properties of these cells and a release of certain of their constituents including endogenous pyrogen, histamine, and serotonin.

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